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ORIGINAL RESEARCH

Restless Legs Syndrome Shows Increased Silent Postmortem Cerebral Microvascular Disease With Gliosis

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BACKGROUND: Patients with restless legs syndrome (RLS) have increased silent microvascular disease by magnetic resonance imaging. However, there has been no previous autopsy confirmation of these magnetic resonance imaging findings. RLS is also frequently associated with inflammatory and immunologically mediated medical disorders. The postmortem cortex in patients with RLS was therefore evaluated for evidence of microvascular and immunological changes.

METHODS AND RESULTS: Ten microvascular injury samples of precentral gyrus in 5 patients with RLS (3 men, 2 women; mean age, 81 years) and 9 controls (2 men, 7 women; mean age, 90 years) were studied by hematoxylin and eosin stains in a blinded fashion. None of the subjects had a history of stroke or neurologic insults. In a similar manner, the following immunohistochemistry stains were performed: (1) glial fibrillary acidic protein (representing gliosis, reactive change of glial cells in response to damage); (2) CD3 (a T-cell marker); (3) CD19 (a B-cell marker); (4) CD68 (a macrophage marker); and (5) CD117 (a mast cell marker). Patients with RLS had significantly greater silent microvascular disease ($P=0.015$) and gliosis ($P=0.003$). T cells were increased in RLS compared with controls ($P=0.009$) and tended to colocalize with microvascular disease ($P=0.003$). Other markers did not differ. There was no correlation between microvascular lesion load and RLS severity or duration.

CONCLUSIONS: Patients with RLS had statistically significantly more silent cerebral microvascular disease and gliosis than controls compatible with previous magnetic resonance imaging studies and with studies showing a link between RLS and hypertension, clinical stroke, and cardiovascular disease. T-cell invasion may be a secondary phenomenon.

Key Words: cortex ■ gliosis ■ microvascular disease ■ restless legs syndrome ■ T cells

Numerous studies over the past decade and more have suggested that restless legs syndrome (RLS) is associated with hypertension, heart disease, and stroke.^{1,2} Magnetic resonance images, for example, have shown that RLS and its accompanying periodic limb movements in sleep are characterized by greater levels of silent cerebral microvascular disease, a known risk factor for stroke.^{3,4} However, there has been no previous autopsy confirmation of these magnetic resonance imaging findings, which is one of the goals of the current study.

Another trajectory of research has been an investigation into the relationship of RLS with inflammatory and immunologic disorders. Many of the comorbid disorders more frequently associated with RLS have an inflammatory or autoimmune diathesis, including multiple sclerosis, rheumatoid arthritis, celiac disease, Crohn disease, inflammatory bowel disease, small intestinal bacterial overgrowth, and, most recently, mast cell activation syndrome.^{5–9} Mast cells play a role in allergy, anaphylaxis, wound healing, angiogenesis, immune tolerance, and defense against pathogens.⁹ The primary goal of the current study is to document with

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CLINICAL PERSPECTIVE

What Is New?

- Previous work has shown that patients with restless legs syndrome (RLS) and its frequently co-occurring periodic limb movements in sleep have more silent cerebral microvascular disease by magnetic resonance imaging; RLS has also been associated with inflammation and autoimmunity.
- To our knowledge there has been no autopsy confirmation of the magnetic resonance imaging findings and no autopsy study looking for autoimmune markers in RLS; in this autopsy study of the cerebral cortex, we show that there is more silent microvascular disease and accompanying gliosis in patients with RLS versus controls.
- T-cell invasion reflective of cellular autoimmunity is also more prominent, and there is no upregulation of CD19 (a B-cell marker), CD68 (a macrophage marker), or CD117 (a mast cell marker).

What Are the Clinical Implications?

- This study is compatible with previous magnetic resonance imaging studies and with studies showing a link between RLS and hypertension, clinical stroke, and cardiovascular disease.
- This study is also compatible with our previous study showing that the treatment of RLS with RLS medications lowers the associated cardiovascular risk.
- Upregulated T-cell levels in patients with RLS could be predisposing factors for development of microvascular disease or a secondary phenomenon.

Nonstandard Abbreviations and Acronyms

AZSAND	Arizona Study of Aging and Neurodegenerative Disorders
RLS	restless legs syndrome

postmortem examination the degree of silent cerebral microvascular disease and gliosis in RLS. The precentral gyrus (motor cortex) was chosen, as neurophysiologic studies have used it as a focus of interest in showing that suprasegmental disinhibition is a major feature of RLS.¹⁰ A secondary goal, based on previous studies demonstrating links between RLS and inflammatory and autoimmunity mechanisms, was to determine if inflammatory markers are also present in RLS cortex, and third, to see if microvascular lesions and inflammatory markers colocalize.

METHODS

Data, Materials, and Code Disclosure Statement

The data that support the findings of this study are available from Paisit Pauksakon, MD, Professor, Associate Director Division of Renal Pathology/ Electron Microscopy and Renal Pathology Fellowship, Division of Neuropathology, Department of Pathology, Microbiology, and Immunology, Vanderbilt University Medical Center (C-23188 Medical Center North, 1161 21st Ave South, Nashville, TN 37232, Tel: 615-322-1350; FAX, 615-322-4840; E-mail: Paisit.Pauksakon@VUMC.org).

A review of the protocol for this study was done by the Vanderbilt University Medical Center Institutional Review Board and was declared “nonresearch” from an Institutional Review Board point of view, and no informed consent was required.

Precentral gyrus (motor cortex) samples were obtained from 5 patients with RLS and 9 controls from the AZSAND (Arizona Study of Aging and Neurodegenerative Disorders) and the Banner Sun Health Research Institute Brain and Body Donation Program.¹¹ Subjects were defined as having RLS if they met the 4 basic clinical criteria for RLS: (1) an urge to move the legs, (2) worsening of symptoms later in the day or at night, (3) worsening of symptoms when lying or sitting, and (4) at least partial and temporary relief by activity. Those who did not meet the criteria for RLS qualified as controls.

All subjects completed a movement disorders examination with questions regarding RLS as well as the Mayo Sleep Questionnaire.¹¹ The subjects chosen all had a long-standing history of RLS. The severity of RLS was rated within ≈3 years of death by the International Restless Legs Severity Scale. Subjects had to meet the criteria above for RLS to be administered the International Restless Legs Severity Scale. The International Restless Legs Severity Scale is a previously validated instrument consisting of 10 questions, where each question is rated from 0 (no RLS symptoms) to 4 (maximum RLS severity), and then the scores for each individual question are added to obtain a total score (0=no RLS symptoms to 40=maximum RLS symptoms).¹²

Exclusion Criteria

In a first round of exclusions, subjects and controls with a known clinical history of previous stroke or a history of Alzheimer disease, dementia, head trauma, Parkinson disease, brain tumor, multiple sclerosis, or other neurodegenerative disorders were excluded from the study. In a second round of exclusions, subjects and controls who had neuropathologic evidence

of stroke, Alzheimer disease, other dementia, head trauma, Parkinson disease, brain tumor, multiple sclerosis, or other neurodegenerative disease were excluded, and at this stage, 1 subject was excluded because of intraparenchymal hemorrhage.

Studies performed on specimens:

1. Microvascular injuries (such as intimal fibrosis of arteries, which correlates with vascular smooth muscle hyperplasia) with hematoxylin and eosin stain.
2. Glial fibrillary acidic protein to look for gliosis, which is a response to brain injury and is a proliferation or hypertrophy of the glial cells, which are supporting cells to the neurons.
3. CD3—look for increase in T cells (cellular immunity).
4. CD19—look for increase in B cells (humoral immunity or antibodies).
5. CD68—look for increase in macrophages (first-line immunologic attack cells).
6. CD 117—look for increase in mast cells (play multiple roles in immunologic mechanisms).
7. Amyloid beta-peptide and tau protein to look for neuritic plaques and neurofibrillary tangles to exclude past brain injury from Alzheimer disease.
8. Ten areas of precentral gyrus of cortex (the motor cortex) were examined in a blinded fashion for each subject and rated 0 to 3+ for each entity of interest by previously described methods.¹³ The total of ratings for each of 10 areas per patient were added to get the sum score and then divided by 10 to get the average. In the above system, 0= no markers of interest; 1=1 marker of interest; 2=2 to 5 markers of interest; and 3=>5 markers of interest. If an immunologic marker showed a statistically significant difference between subjects with RLS and controls, colocalization of immunologic markers and microvascular disease was determined by the number of immunologic markers per vascular cross section by the same methodology.

A detailed description of tissue processing and immunohistochemistry including light microscopy and immunohistochemical studies can be found in Data S1.

Statistical Analysis

A stepwise analytic approach was conducted. Primary variables were microvascular lesion load and gliosis. Secondary variables were inflammatory markers. Tertiary variables were RLS duration and severity and past medical history. The Mann-Whitney *U* test was applied to assess group differences for continuous variables, and Fisher's exact test was applied for categorical variables. The correlation of RLS duration and

RLS severity to microvascular lesion load was performed by Spearman's rho. Post hoc, we calculated the Hedges' *g*, which is an effect-size measure, and calculated the power of that effect size.

Statistical analyses were performed with Statistica version 13 (TIBCO Software Inc, Palo Alto, CA).

RESULTS

We evaluated 5 RLS patients (3 men and 2 women; mean age, 81 years; range, 65–93 years) and 9 controls (2 men and 7 women; mean age, 90.2 years; range, 78–99 years). Table 1 gives the demographic information for subjects and controls in the study. There was no statistically significant difference in age or sex between patients and controls (Table 1). Of the 5 subjects who had RLS, 1 subject was on hydrocodone, 1 on clonazepam, 1 on both ropinirole and tramadol for treatment of RLS, and 2 subjects were on no RLS treatment (Table 1).

Primary Variables

Table 2 gives individual subject information for the markers of interest. There was a significant difference in the extent of silent cerebral microvascular disease ($P=0.015$) (Figure 1 and Table 3) and the level of gliosis ($P=0.003$) (Figure 2 and Table 3) in patients with RLS compared with controls. All patients and controls were negative for amyloid beta-protein and tau protein (Tables 2 and 3).

Secondary Variables

T cells were also increased in RLS compared with controls ($P=0.009$) (Figure 3 and Table 3). T cells tended to colocalize with microvascular disease, and the colocalization was significantly greater in patients with RLS than controls ($P=0.003$) (Table 3).

By Mann-Whitney *U* test there was no significant difference between patients and controls in CD68 ($U=22$; $Z=0$; $P=1.0$) or CD117 ($U=18$; $Z=-0.21958$; $P=0.825$) (Table 3). There was no evidence of CD19 B-cell antibody production in patients or controls (Tables 2 and 3).

Tertiary Variables

Given the small sample size and the fact that not all the secondary variables differed significantly between groups, further analysis should be considered exploratory. There was no significant correlation with Spearman's rho between RLS duration ($r_s=0.72$; $P=0.17$) or severity ($r_s=-0.41$; $P=0.49$) and microvascular lesion load (Table 1). Chi-square analysis did not find a significant imbalance of cardiovascular disease (myocardial infarction, congestive heart

Table 1. Clinical Characteristics of the Patients With RLS and Controls

Patient No.	Age, y	Sex	RLS Duration, y	RLS Severity Score	Time From IRLS Score to Autopsy	RLS Treatment
1	93	F	2	4	3 mo	None
2	79	M	8	12	39 mo	Hydrocodone
3	80	M	13	15	5 mo	Clonazepam
4	65	M	3	28	24 mo	Ropinirole tramadol
5	88	F	27	9	16 mo	None
Control no.	Age, y	Sex
1	88	F
2	78	F
3	87	F
4	91	M
5	84	F
6	96	M
7	99	F
8	93	F
9	96	F

RLS severity score is the IRLS, a previously validated scale in which 10 questions are each rated from 0 (no RLS symptoms) to 4 (maximum RLS symptoms) and then totaled (range, 0–40). RLS treatment is indicated as treatment at the time of death. There is no statistically significant difference in age ($U=11$; $P=0.14$) or sex ($P=0.2657$ by 2-tailed Fisher's exact test) between patients and controls. There was not a statistically significant correlation by Spearman's rho between RLS duration ($r_s=-0.72$; $P=0.17$) or severity ($r_s=-0.41$; $P=0.49$) and microvascular lesion load. IRLS indicates International Restless Legs Syndrome Severity Scale; and RLS, restless legs syndrome.

failure, coronary artery disease, cardiac valve disease, cardiac arrhythmia, cardiomegaly) between the RLS group (3/5 subjects) and the control group (8/9 subjects) group. By chi-square there was also no significant difference in other types of cardiovascular risk factors between the groups such as diabetes mellitus (1/5 versus 1/9), hypertension (2/5 versus 7/9), or hyperlipidemia (2/5 versus 6/9). In addition, chi-square did not show an imbalance of cancer between the RLS group (3/5 subjects) and the controls (3/9 subjects).

DISCUSSION

The major finding in this autopsy-based study was that in subjects without a previous history of stroke or any other neurologic disease, silent ischemic cerebrovascular disease and gliosis in the motor cortex were more common in RLS subjects than controls. These findings support previous magnetic resonance imaging findings of an increase in leukoarteriolar cerebrovascular disease in patients with RLS and its allied condition periodic limb movements in sleep compared with controls.^{3,4}

Additionally, T-cell infiltration was more common in subjects with RLS than in controls. These findings also support previous findings of a link between RLS and inflammation.⁵ The immunologic alteration in the current study suggests that cellular immunity may be altered in RLS, given the increased T-cell invasion (CD3) we found as opposed to humoral

or antibody immunity (CD19—B cells), the first line immunologic attack system (CD68—macrophages), or allergy/anaphylaxis (CD117—mast cells). On the other hand, these data are also compatible with the normal response of the brain to ischemia. At the lesion border in acutely ischemic areas, there is initially an invasion of polymorphonuclear leukocytes followed soon after by T lymphocytes, B lymphocytes, mast cells, and activation of microglia. Over the next few days there is an invasion of macrophages that eventually become the predominant inflammatory cell, but evidence also exists that T cells are the leukocytic lineage that may persist for the longest time after ischemia.^{14–19} Leukocytic activity during acute cerebral ischemia may contribute to the inflammatory process and to neuronal injury but may also later provide protective and reparative functions to the damaged area. Increased T-cell activation in our patients with RLS may simply be compatible with the increased microvascular lesion load in these subjects, and may represent a secondary adaptive neuroprotective response to prior ischemia. On the other hand, we cannot exclude the possibility that the T-cell presence represents an immune manifestation of RLS. One would assume that the microvascular lesions have been present for some time, long after one would expect macrophage and mast cell invasion. Macrophages and mast cells were present in both our patients with RLS and controls, but no difference was noted in the degree to which these cell types were present between the 2 groups. The

Table 2. Average Scores per Subject for Microvascular Injury, CD3, CD3 Microvascular Colocalization Score, CD19, CD68, CD117, GFAP, Amyloid Beta Peptide, Tau Protein in Subjects With RLS and Control Subjects

Patients With RLS	Microvascular Injury Score	CD3 Score	CD3 Microvascular Colocalization Score	CD19 Score	CD68 Score	CD117 Score	GFAP Score	Amyloid Beta-Peptide Score	Tau Protein Score
1	3.0	0.5	1.1	0	0.1	2.3	3.0	0	0
2	3.0	0.2	2.1	0	0.1	2.1	2.3	0	0
3	0.4	0.1	1.1	0	0.1	0.9	1.8	0	0
4	2.9	0.2	2.3	0	1.1	2.0	2.2	0	0
5	2.0	0.5	2.1	0	0.1	1.2	2.2	0	0
Avg	2.26	0.3	1.7	0	0.3	1.7	2.3	0	0
Control Subjects	Microvascular Injury Score	CD3 Score	CD3 Microvascular Colocalization Score	CD19 Score	CD68 Score	CD117 Score	GFAP Score	Beta-Peptide Score	Tau Protein Score
1	0	0	0.4	0	0.1	1.5	0	0	0
2	0.1	0	0.3	0	0.1	1.5	0	0	0
3	0.2	0	0.2	0	0.1	2.0	0.2	0	0
4	1.9	0.2	0.9	0	0.1	2.1	1.5	0	0
5	0	0.2	0.9	0	0.1	1.9	0.2	0	0
6	0.9	0	0.9	0	0.2	1.8	0.4	0	0
7	0.1	0	1.0	0	0.1	1.6	0	0	0
8	1.9	0	0.6	0	0.8	1.9	1.6	0	0
9	1.1	0	0.3	0	0.1	...	0.9	0	0
Avg	0.688	0.044	0.470	0	0.188	1.788	0.533	0	0

Ten areas of precentral gyrus of cortex (the motor cortex) were examined in a blinded fashion for each subject and rated 0 to 3+ for each entity of interest. The total of ratings for each of 10 areas per patient were added to get the sum score. Each number in the table is average, that is, the sum score divided by 10. GFAP indicates glial fibrillary acidic protein; and RLS, restless legs syndrome.

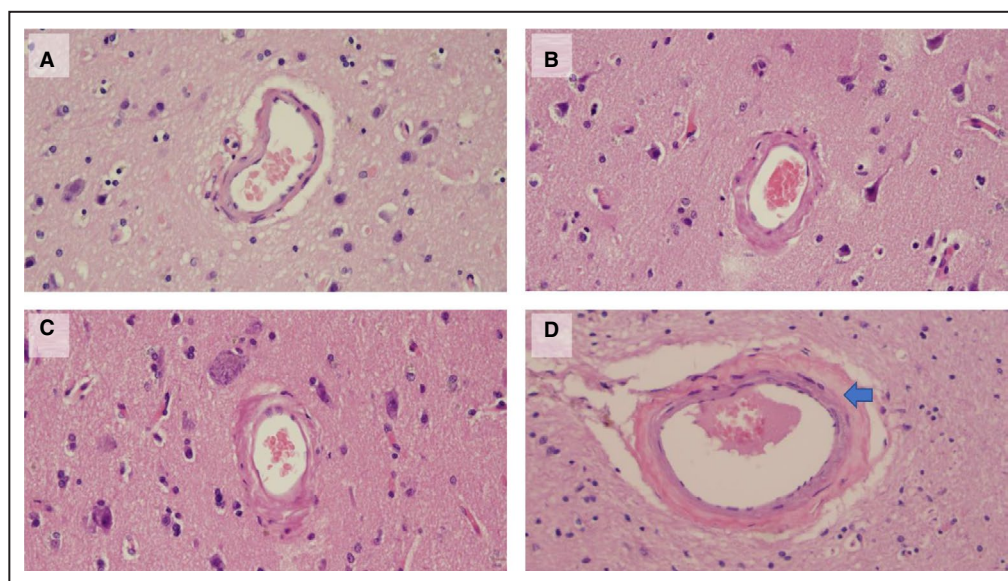


Figure 1. Examples of level of microvascular injury score in controls (A and B) and patients with restless legs syndrome (RLS) (C and D).

A, Score 0; B, score 1; C, score 2; and D, score 3. There is marked increase in microvascular injury in patients with RLS characterized by intimal fibrosis of artery (arrow). Hematoxylin and eosin stain, $\times 200$. Patients with RLS had significantly greater silent microvascular disease than controls ($P=0.015$). See details in Tables 2 and 3.

fact that T cells are more prominent in the subjects than the controls suggest that the microvascular lesions observed are chronic rather than acute since T cells persist in the context of vascular insult long after other cell types have diminished or disappeared.^{14–19} Other cell types may have diminished to the point that previous differences between subjects and controls, in the degree to which these cell types were present, are no longer detectable.

The major limitation to this study was the small sample size. Autopsy cases of subjects with RLS having little to no other neurologic abnormality clinically or neuropathologically is very rare. As our methodology excluded subjects with other neurologic insults, our sample size was of necessity small. Another limitation was the possibility that RLS treatment could cause the microvascular disease, but this is unlikely given a previous study showing that treatment ameliorates

Table 3. Statistical Comparison Between RLS and Control Group

	RLS		Control		Test Statistic	Hedges' g	Power, %
	M \pm SD	Mdn (QR)	M \pm SD	Mdn (QR)			
Microvascular injury score	2.3 \pm 1.1	2.9 (1)	0.7 \pm 0.8	0.2 (1.0)	$U=4, P=0.015$	1.6 \pm 0.6	77.2
CD3 score	0.3 \pm 0.2	0.2 (0.3)	0.0 \pm 0.1	0.0 (0.0)	$U=4, P=0.009$	2.0 \pm 0.6	90.4
CD3 MC score	1.7 \pm 0.6	2.1 (1)	0.6 \pm 0.3	0.6 (0.6)	$U=0, P=0.003$	2.4 \pm 0.7	97.8
CD19 score	0	0	0	0			
CD68 score	0.3 \pm 0.4	0.1 (0)	0.2 \pm 0.2	0.1 (0.0)	$U=22, P=1$	0.3 \pm 0.5	8.5
CD117 score	1.7 \pm 0.6	2.0 (0.9)	1.8 \pm 0.2	1.9 (0.4)	$U=18, P=0.825$	-0.2 \pm 0.5	6.9
GFAP score	2.3 \pm 0.4	2.2 (0.1)	0.5 \pm 0.6	0.2 (0.9)	$U=0, P=0.003$	3.1 \pm 0.8	99.9
Amyloid beta-peptide score	0	0	0	0			
Tau protein score	0	0	0	0			

Patients with RLS had significantly greater silent microvascular disease ($P=0.015$) and gliosis as demonstrated by GFAP ($P=0.003$). T cells, as demonstrated by CD3, were increased in RLS compared with controls ($P=0.009$) and tended to colocalize with microvascular disease ($P=0.003$). Other markers did not differ. The Hedges' g statistic indicates the effect size for the difference between means. Power=1- β error probability, for 2-tailed α . CD3 MC score indicates T cell (CD3) microvascular colocalization; GFAP, glial fibrillary acidic protein; M, mean; Mdn, median; QR, quartile range; and RLS, restless legs syndrome.

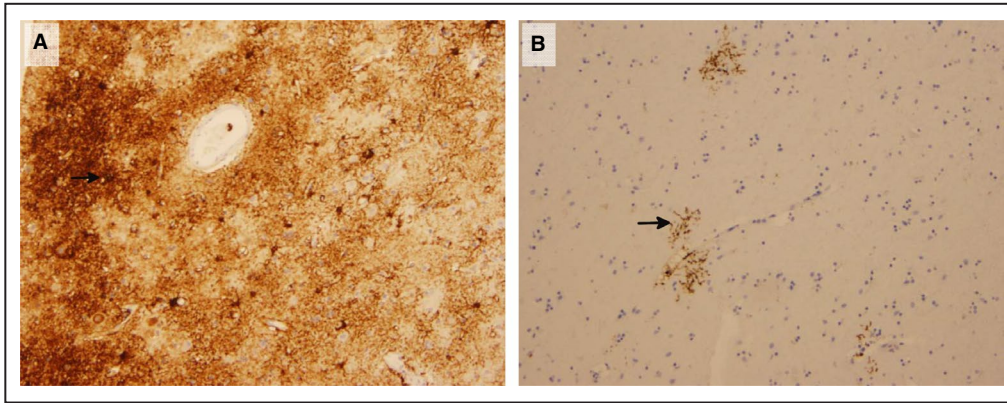


Figure 2. There is a marked increase in gliosis as demonstrated by GFAP (glial fibrillary acidic protein) in patients with restless legs syndrome (RLS) (A) compared with controls (B) ($P=0.003$). The arrow points to perivascular astrocytes (anti-GFAP stain, $\times 200$). See details in Tables 2 and 3.

the likelihood of developing future cardiovascular disease.²⁰

Considerations for future research into the immunopathogenesis of RLS include flow cytometry to look for differences in the T-cell subtypes between subjects with RLS and controls as well as RNA sequencing relevant to T-cell functioning.

CONCLUSIONS

Patients with RLS had statistically significantly more silent cerebral microvascular disease and gliosis than controls, compatible with previous magnetic resonance imaging studies and with studies showing a link between RLS/periodic limb movements in

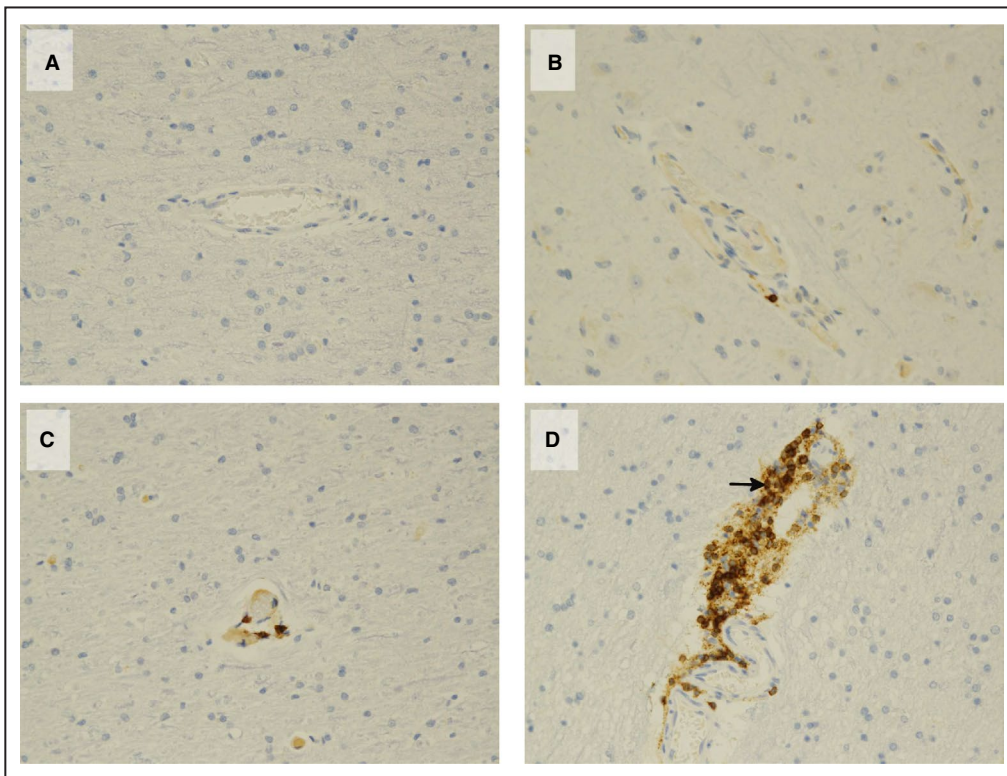


Figure 3. Examples of level of T-cell (CD3) staining score in controls (A and B) and patients with restless legs syndrome (RLS) (C and D).

A, Score 0; B, score 1; C, score 2; and D, score 3. There is marked increase in CD3 staining in patients with RLS (arrow). Anti-CD3 stain, $\times 200$. T cells, as demonstrated by CD3, were increased in RLS compared with controls ($P=0.009$) and tended to colocalize with microvascular disease ($P=0.003$). See details in Tables 2 and 3.

sleep and hypertension, clinical stroke, and cardiovascular disease. Upregulated T-cell levels in patients with RLS could be predisposing factors for development of microvascular disease or a secondary phenomenon.

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Supplementary Material

Data S1

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SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Tissue processing and Immunohistochemistry

Light microscopy

Briefly, brain tissues were fixed in buffered formaldehyde, dehydrated in graded alcohols, and embedded in paraffin using standard techniques. Serial 5 μm -thick sections were cut and stained with hematoxylin and eosin reagent.

Immunohistochemical studies

For CD3 detection, 5 μm -thick paraffin-embedded sections were cut, deparaffinized, rehydrated. Slides were placed on the Leica Bond Max IHC stainer. All steps besides dehydration, clearing and coverslipping are performed on the Bond Max. Slides were deparaffinized. Heat induced antigen retrieval was performed on the Bond Max using their Epitope Retrieval 2 solution for 20 minutes. Slides were incubated with Ready-To-Use anti-CD3 (Cat# MM150-10, StatLab McKinney, TX) for one hour. The Bond Polymer Refine system was used for visualization. Slides were the dehydrated, cleared and coverslipped.

For CD19, CD68, and CD117 detection, 5 μm -thick paraffin-embedded sections were cut, deparaffinized, rehydrated. Slides were placed on the Leica Bond Max IHC stainer. All steps besides dehydration, clearing and coverslipping were performed on the Bond

Max. Slides are deparaffinized. Heat induced antigen retrieval was performed on the Bond Max using their Epitope Retrieval 1 solution for 20 minutes. Slides were incubated with Ready-To-Use anti-CD19 (PA0843, Leica Biosystems, Newcastle, Tyne, NE, UK), Ready-To-Use anti-CD68 (MM36-10, StatLab, McKinney, TX), and Ready-To-Use anti-CD117 (PA0007, Leica Biosystems, Newcastle, Tyne, NE, UK) for 15minutes. The Bond Polymer Refine detection system was used for visualization. Slides were the dehydrated, cleared and coverslipped.

For GFAP detection, 5 µm-thick paraffin-embedded sections were cut, deparaffinized, rehydrated. Slides were placed on the Leica Bond Max IHC stainer. All steps besides dehydration, clearing and coverslipping are performed on the Bond Max. Slides were deparaffinized. Heat induced antigen retrieval was performed on the Bond Max using their Epitope Retrieval 2 solution for 5 minutes. Slides were incubated with Ready-To-Use anti-GFAP (PA0026, Leica Biosystems, Newcastle, Tyne, NE, UK) for 15minutes. The Bond Polymer Refine detection system was used for visualization. Slides were the dehydrated, cleared and coverslipped.

Normal lymphoid tissue was used as a positive control for CD3, CD19, and CD68.

Normal gastric tissue was used as a positive control for CD117. Brain tissue with reactive gliosis was used as a positive control for GFAP. Negative controls were done omitting primary antibody.